Advances in Ex Vivo Modified Cell Therapies

1. Development of an Optimized Lentiviral Transduction Process for *Ex Vivo* CD34+ Hematopoietic Stem Cell Gene Therapy Drug Product Manufacture

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Gene therapy using transplantation of autologous *ex vivo* gene modified $CD34^+$ hematopoietic stem cells (HSC) as an approach to treat a range of monogenic disorders, is now recognized for its transformative potential through several clinical studies and regulatory approvals. Despite significant advances in the commercialization of gene therapies, the main barriers to patient accessibility are the current limited capacity for manufacture of GMP-grade lentiviral (LV) vectors, and the corresponding substantial costs incurred by production of gene and cell therapies. This is further compounded by the need to manufacture higher numbers of gene modified HSCs for adult patients such as those suffering from X-Linked Chronic Granulomatous Disease (X-CGD), and for indications with larger patient cohorts. Reducing vector requirements and cost of goods therefore presents a key challenge in commercializing gene therapies. Application of transduction enhancers enables the use of lower quantities of LV vector to achieve the same output of gene modified cells. Several enhancer compounds are already routinely applied in clinical gene and cell therapy manufacture to improve the viral transduction process at various cellular levels, such as viral attachment, vector entry, and genome integration. To develop an optimized protocol for LV transduction of HSCs, we have screened over 20 commercially available and novel candidate compounds for enhancement activity, when applied individually or in combination to target distinct viral transduction pathways. Our comprehensive survey of improvements in transduction efficiency (TE) and vector copy number (VCN) achievable by these enhancers was conducted with both scale-down high-throughput and clinical-scale transduction processes for HSC gene therapy drug product manufacture, using clinical-grade therapeutic LV vectors. The most potent enhancer combinations were then assessed for compatibility with other known transduction culture process modifications, to develop an optimized protocol for HSC transduction. Enhancer treated HSCs were subject to extensive *in vitro* and *in vivo* characterization, including RNAseq transcriptional profiling and competitive engraftment studies in mice. Here we describe J-Boost, a representative compound from a novel class of transduction enhancers (diblock copolymers, PCT/US20/56123) which facilitates viral entry. When used in concert with Protamine Sulphate (PS) and high-density cultures, J-Boost results in up to ~9 fold increases in VCN and ~4 fold increases in TE, enabling 50-70% reduction in LV vector for HSC transduction to achieve desired target drug product profiles. Transduction is further enhanced by use of Retronectin™ coated culture vessels. Our results demonstrate that J-Boost/ PS enhancers are largely inert, inducing minimal alteration of HSC gene expression, HSC phenotype and multilineage progenitor function, while generating gene modified HSCs with comparable qualitative and quantitative HSC engraftment potential in NSG mice. Importantly, we show compatibility of this optimized transduction protocol with therapeutic LV vectors and manufacture methods currently in use for drug products under development for Beta-thalassemia, Mucopolysaccharidosis I (MPS I) and X-CGD. Demonstration of the safety, efficacy and comparability of this optimized transduction protocol validates its potential for clinical application and the achievable reduction in vector usage and manufacturing costs.

2. Non-Viral Integration of Large Cargo in Primary Human T Cells by CRISPR/Cas9 Guided Homology Mediated End Joining

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Adoptive cell therapy (ACT) using genetically engineered immune cells, such as CAR-T cells, holds tremendous promise for the treatment of advanced cancers. Current methods for the manufacturing of these cells rely on the use of viral vectors, greatly increasing manufacturing time, expense, and complexity. Furthermore, these viral vectors integrate in a non-site specific manner reducing functionality and raising safety concerns. Here we describe methods for efficient CRISPR-based, non-viral engineering of primary human T cells that overcome key limitations of previous approaches, namely DNA-induced toxicity and low efficiency integration of large genetic cargos. By synergizing temporal optimization of delivery, reagent composition, and integration mechanism, we achieve targeted knockin of cargo ranging from 1 to 3 kilobases at rates of up to 70% at AAVS1 (Figure 1), with post-editing cell viability of over 80%; efficiencies nearing those of viral vector platforms. Notably, approaches utilizing homology mediated end joining (HMEJ) and shorter homology arms (48bp) consistently outperformed those using longer 1kb homology arms and traditional homologous recombination. Off-target editing and integration were evaluated using GUIDE-seq and targeted locus amplification (TLA), respectively. As proof of concept, we engineered CAR-T cells and transgenic TCR T cells using a splice acceptor gene construct and gRNA specific to the TRAC locus, such that the CAR or transgenic TCR is expressed under the control of endogenous TRAC regulatory elements. Using this approach we consistently achieved integration

rates of over 20% for CAR-T cells and over 25% for TCR transgenic T cells (Figure 2). Additional optimizations, including culturing cells with anti-CD3 and anti-CD28 antibodies immediately following electroporation, further increased integration, averaging 39% for CAR templates. Furthermore, we demonstrate that these cells remain highly functional, retaining low expression of exhaustion markers, excellent proliferation and cytokine production capacity, and

potent anti-tumor cytotoxicity equal to or better than cells generated using a viral vector. Most importantly, these methods result in minimal-to-undetectable off-target editing and are readily adaptable to cGMP compliant and clinical-scale manufacturing. This non-viral gene engineering protocol offers a realistic, near-term alternative to the use of viral vectors in the production of genetically engineered T cells for cancer immunotherapy, offering immense potential for reducing manufacturing time, cost, and complexity compared to viral vectors without compromising cell expansion or function, while potentially increasing safety and efficacy via targeted integration.

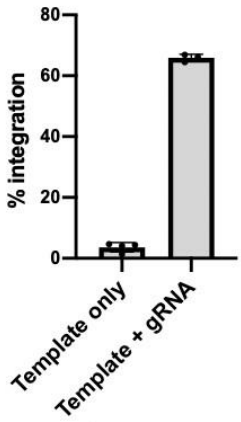

Figure 1. Expression of GFP in primary human T cells 8 days after transfection with Cas9 mRNA and a DNA minicircle template with 48bp of homology to AAVS1 encoding splice acceptor-GFP in the presence or absence of AAVS1 gRNA. GFP expression measured by flow cytometry.

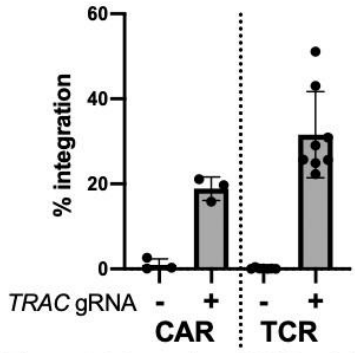

Figure 2. Expression of CAR or TCR in primary human T cells 7 days after transfection with Cas9 mRNA and a DNA minicircle template targeting TRAC and encoding CAR or TCR in the presence or absence of TRAC gRNA. Expression measured by flow cytometry.

3. Epigenetic Modulation of Aging to Increase CAR-T Cell Fitness

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Background: CAR-T cell therapy has had incredible clinical success in the treatment of hematological malignancies. However, very limited activity against solid tumors has been achieved so far, despite targeting a variety of antigens and tumor types. Here we show the impact of reducing cellular senescence in CAR-T cells to improve T cell fitness. Increasing evidence shows that immunosenescence is an important state of T cell dysfunction distinct from exhaustion; solid

tumors strongly induce senescence as a key strategy to evade immune surveillance and sustain a suppressive tumor microenvironment. Notably, the *ex vivo* manufacturing process of CAR-T cells also induces senescence extremely quickly; 15 days of T cell expansion age cells 30 years, as measured by telomere length, T cells differentiation and CDKN2a mRNA levels. Here we show that rejuvenating T cells by blocking cellular aging at the epigenetic level targeting USP16 is a powerful strategy to improve T cell fitness and clinical outcomes. Methods and Results: T cell aging is a very early phenomenon upon T cell activation, and it is drastically accelerated during ex vivo CAR-T manufacturing, resulting in a huge reduction of their ability to expand and kill cancer cells. In line with this hypothesis, we demonstrated that reducing cellular senescence increases CAR-T cell functions both in vitro and in vivo. USP16 is a deubiquitinating enzyme responsible for the removal of ubiquitin moieties from histone H2AK119, increasing chromatin accessibility to pro-senescent programs. Here, we show that targeting USP16 by means of a shRNA co-expressed within a CD19 or a GD2.CAR construct reduces T cell aging and increases stem cell memory (Tscm) frequency during manufacturing, without affecting proliferation. USP16 modulation also results in increased killing, polyfunctionality, and expansion upon in vitro stimulation with tumor cells. Notably, the delay of cellular senescence induces long-lasting cellular fitness as T cells are less exhausted upon multiple tumor challenges. Finally, T cells rejuvenated by USP16 modulation, show a strong increase in anti-tumor activity in an in vivo model of leukemia and neuroblastoma. **Conclusions:** Preventing T cell senescence by modulating the expression of USP16 increases self-renewal and antitumor activity, significantly improving the efficacy of CAR-T therapy. Development of small molecules against USP16 could offer a viable solution to improve T cell fitness during manufacturing.

4. Effective and Efficient Intracellular Delivery Achieved with the Cell Squeeze® Technology Enables Rapid, Scaled, and Reproducible Production of Cell Therapies

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Personalized cellular therapies, especially in cancer, have grown in number over the last five years. Cell therapies require precise biological engineering using cargos which are intracellularly delivered. However, conventional approaches for delivering materials into cells are limited in the types of biology they can engineer and have manufacturing challenges with regards to scale and reproducibility. The Cell Squeeze® technology uses microfluidic chips with constrictions to deform cells resulting in the effective intracellular delivery of molecules to cells while preserving cell health and function. This technique enables numerous opportunities to reproducibly generate new classes of cell therapies at scale. We have implemented the Cell Squeeze® process for cGMP and aseptic manufacturing. Our system is appropriate for use in a Grade B cleanroom utilizing a single-use sterile disposable kit containing multiple microfluidic chips. The Cell Squeeze® manufacturing system can accommodate up to 20 billion cells for processing in 2 minutes or less. Cell viability at manufacturing scale is >90% and the percent of the cell population delivered is >80%. This system is currently being used to manufacture drug product in a Phase 1 clinical trial using peripheral blood mononuclear cells (PBMCs) to generate antigen presenting cells as a cellular vaccine. To further enhance accessibility of SQZ Biotechnology cell therapies, we are also developing a point-of-care manufacturing system with the potential to manufacture cell therapies for same-day dosing. Our advances in cell therapy manufacturing will potentially enable multiple therapeutics across oncology, infectious disease, and immune disorders to be developed and delivered to a broad patient population.

5. Sequential CRISPR-Mediated Engineering and Clonal Banking for the Generation of Multiplexed Engineered Master Pluripotent Cell Lines for the Mass Manufacture of Offthe-Shelf Immune Cells Targeting Solid Cancers

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Chimeric antigen receptor (CAR) T and NK cell therapies have shown great promise in treating blood malignancies, but tackling solid tumors is hindered by a shortage of targetable tumor-specific antigens, antigen escape, and poor activity of expanded patient-derived immune cells. While a multiplexed engineering approach can arm immune cells to address these obstacles, engineering patient- or donor-derived NK and T cells often leads to inconsistent, heterogeneous, and costly products. We describe here a platform to enable complex genetic engineering and precise targeting of independent loci through sequential rounds of CRISPR-mediated editing, single cell cloning, screening and banking of induced pluripotent stem cells (iPSCs) leading to the generation of multiplexed engineered master iPSC clones. Using this platform, we are developing a master iPSC bank that is uniformly engineered with four anti-tumor modalities. A core of three edits are first introduced and include: i) IL-15 receptor fusion protein (IL-15RF) for enhanced NK cell activity, ii) high affinity non-cleavable CD16 (hnCD16) for enhanced antibody-dependent cellular cytotoxicity, and iii) CD38 deletion for enhanced NK cell function. The fourth edit, CAR targeting the HLA-I related molecules MICA and MICB (MICA/B), is engineered

next in a second locus for pan-tumor targeting. Challenges with building such a complex cellular therapy include genomic instability and off-target editing that could be introduced during CRISPR/nuclease-based engineering of multiple loci, and during the multiple

rounds of single cell cloning, expansion, and banking. To reduce offtarget edits that could be caused by simultaneously targeting two loci, we performed sequential engineering instead, targeting one locus at a time to build a more complex master iPSC line. In the first step of the engineering process, iPSCs were engineered with the three core antitumor modalities by targeting an IL-15RF-hnCD16 cassette into the CD38 locus, resulting in complete CD38 gene disruption. The CD38 gene was targeted with fully characterized gRNA and donor plasmid containing the IL15-RF-hnCD16 cassette. Engineered iPSCs were cloned from single cells and clones were screened and fully tested before and after banking. Out of 257 iPSC clones screened, 88 clones (34%) had the IL-15RF-hnCD16 transgenic cassette targeted specifically into the CD38 gene. Evaluation of genomic stability of the selected clones after banking revealed that 12 of the 15 selected clones maintained genomic stability as determined by G-banded karyotyping, and 10 clones out of 11 tested showed no genomic copy number variations as determined by a genome-wide SNP microarray analysis. Banked iPSC clones were further evaluated for pluripotency and propensity to differentiate into NK cells which were further tested for phenotype and function. The best performing clone was then selected for a second round of genetic engineering, whereas CAR-MICA/B is inserted into a safe harbor locus at the single cell level to create the final master iPSC clone containing the three core edits and the CAR-MICA/B. The generated engineered iPSC subclones are currently being screened for specific targeting of the CAR-MICA/B into the safe harbor locus and other critical quality attributes (including maintenance of pluripotency and genomic stability) to nominate the final iPSC master cell bank. This master cell bank will serve as the starting material for clinical investigation of FT536, an off-the-shelf pan-cancer targeting CAR NK cell immunotherapy with potential therapeutic application to multiple hematopoietic and solid tumors.

6. Generation of Engineered Tregs (EngTregs) from Umbilical Cord Blood Derived CD4⁺ T Cells via HDR-Dependent *FOXP3* Gene Editing

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Advances in Ex Vivo Modified Cell Therapies

cell doses and maintaining cell purity and Treg stability. We previously developed gene engineered Tregs (EngTregs) from PB-derived CD4⁺ T cells as an alternative source for Treg cell therapies. This approach utilizes co-delivery of a *FOXP3*-targeting designer nuclease and an rAAV homology-directed-repair (HDR) donor template designed to introduce a gene cassette containing MND promoter and cis-linked LNGFRt surface tag into the *FOXP3* locus. The resulting enforced and stable expression of FOXP3 reprograms HDR-edited cells to acquire a Treg-like phenotype and suppressive function in vitro and in vivo in xeno-GvHD mouse models. This approach permits clinical-scale production of highly purified EngTregs for potential therapeutic use. The naïve phenotype of CD4⁺ T cells in UCB suggests a potential proliferation and potency advantage over T cells in adult PB as a source for EngTregs production. Further, HDR editing in UCB T cells might provide the capacity to generate multiple cell doses for shortterm allogeneic cell therapies. In the current study, we evaluated the feasibility of using UCB-derived CD4⁺ T cells to generate EngTregs. We found that UCB-derived CD4⁺ T cells could be edited at a high efficiency. Edited cells could also be efficiently enriched by the surface LNGFRt selection marker. Notably, the expansion methods optimized for PB-derived EngTregs (rapamycin treatment, high dose of IL-2 and G-rex culture) did not yield comparable cell viability or quantity for UCB-derived EngTregs. Therefore, we established alterative protocols for cell expansion; approaches that significantly improved the yield and viability of the UCB EngTregs products. In parallel with these studies, we also tested an alternative editing strategy designed to introduce a heterodimeric, chemically-induced signaling complex (CISC; that mimics IL-2 signaling in response to an exogenous dimerizer) upstream of the *FOXP3* gene. UCB derived EngTregs cells products (including LNGFRt+ or CISC+ EngTregs, respectively) exhibited high purity, stable FOXP3 expression, consistent expression of key Treg markers and limited expression of proinflammatory cytokines upon stimulation. Additionally, we tested whether UCB-derived EngTregs could suppress allogeneic effector T cells in the xeno GvHD mouse model. Our preliminary data showed that the UCB-derived EngTregs have suppressive capabilities against HLA-mismatched allogeneic effector T cells in NSG recipient mice. In summary, our data demonstrate a robust capacity to engineer UCB-derived T cells leading to generation of EngTregs that exhibit a stable Treg phenotype and function. Additional optimization of cell expansion is likely to provide cell yields that enable UCB to be a viable future cell source for EngTregs.

7. VOR33: A Clinic-Ready CRISPR/Cas9 Engineered Hematopoietic Stem Cell Transplant for the Treatment of Acute Myeloid Leukemia

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Regulatory T cells (Tregs) play a crucial role in peripheral immune tolerance and homeostasis. Adoptive transfer of Tregs is being pursued in clinical trials for autoimmune diseases and for bone marrow and solid organ transplantation. Current Treg trials utilize ex-vivo expanded thymic Tregs (tTregs) derived from either peripheral blood (PB) or umbilical cord blood (UCB); however, this approach must overcome technical challenges that include manufacturing adequate

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Introduction: AML is the most common form of adult acute leukemia, with median 5-year survival rate <30%. Allogeneic hematopoietic cell transplant (HCT) has long been the standard of care for high-risk patients (pts), with >3500 transplants performed annually in the US. There is unmet need for new treatments in ~40% of pts who relapse. With existing targeted therapies, cell surface marker expression between cancer and normal cells is not differentiated enough to limit "on-target, off-tumor" toxicity. Antigens (Ag) (eg, CD33) expressed on normal myeloid cells and/or progenitors (Levine et al. 2015) confer dose-limiting toxicity of Ag-directed therapies in AML. To unlock the full potential of targeted treatments, we create treatmentresistant hematopoietic stem cells (HSCs) by genetically ablating CD33 from healthy, HLA-matched (10/10) donor HSCs followed by HCT, creating a target Ag-negative hematopoietic system. The reconstituted hematopoietic compartment of pts receiving CD33-null cells will be resistant to cytotoxicity induced by Mylotarg™, an antiCD33 monoclonal antibody conjugated with cytotoxic calicheamicin. Human CD33 null hematopoietic cells show no impairment of function and are resistant to CD33-targeted therapies (Borot et al. 2019; Humbert et al. 2019; Kim et al. 2018). Notably, this is consistent with natural genetic evidence of CD33 null humans with no deleterious phenotype (gnomad.broadinstitute.org/). Here, we describe the preclinical data and process scale-up of the CD33-null HSC graft (VOR33) for a first-in-human clinical trial. **Methods/Results:** The manufacturing process yielded clinically relevant doses of VOR33 (>3x10^6 viable CD34+ cells/kg) under GMP-like conditions with GMP-appropriate reagents. CD34+ cells, isolated from G-CSF and plerixafor mobilized peripheral blood leukapheresis products, were edited using CRISPR/Cas9 to disrupt *CD33* gene. At scale, we routinely achieved gene knockout of >70% (90% biallelic) with no loss of cell viability. Cells differentiated from VOR33 displayed normal myeloid markers, phagocytosis potential and induction of inflammatory cytokines equivalent to unedited (CD33+) control cells. Phenotypic and functional characterization revealed no difference in frequency of long-term HSCs in VOR33 vs unedited controls. Pharmacology studies using NOD/SCID-gamma mice, with VOR33 cells manufactured under GMP-like conditions, showed normal long-term engraftment (16-week bone marrow chimerism of 83.1±9.0% vs 87.9±7.3% in control group) and multilineage differentiation. In addition, we observed persistence of VOR33 gene editing and preservation of indel species distribution after 16 weeks, indicating no counterselection or clonal expansion of CD33-null cells. Importantly, we found loss of CD33 protein conferred selective protection to VOR33-derived myeloid cells vs Mylotarg *in vitro* (>65-fold) and *in vivo* (>60-fold). In our GLP toxicology study of >40 tissues, we saw no tumorigenicity or notable changes in toxicology parameters. In-depth genotoxicity analyses were carried out with a subset of scaled-up manufactured lots of VOR33, including those used for toxicology and pharmacology studies. Deep sequencing of 2369 genomic sites, by homology-dependent and independent methods, revealed no off-target editing. **Conclusion:** These studies set the stage for initiation of, as well as evaluation of safety and efficacy in, a multicenter first-in-human clinical trial of VOR33 in pts with AML.

Cancer - Oncolytic Viruses

8. Preclinical Toxicology Assessment of an Oncolytic Measles Virus Armed with *H. pylori* Immunostimulatory Bacterial Antigen in Preparation for a Phase I Trial in Breast Cancer Patients

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Introduction: Despite recent therapeutic advances, metastatic breast cancer (MBC) remains incurable. Engineered measles virus (MV) strains based on the attenuated MV Edmonston vaccine platform have demonstrated significant oncolytic activity against solid tumors. *Helicobacter pylori* neutrophil-activating protein (HP-NAP) is responsible for the robust inflammatory reaction in the gastroduodenal mucosa during infection. NAP attracts and activates immune cells at the site of infection inducing expression of inflammatory mediators. Our team engineered an MV strain expressing the secretory form of NAP (MV-sNAP) that exhibits anti-tumor and immunostimulatory activity in human breast cancer xenograft models. In this study, we investigated the biodistribution and toxicity of MV-sNAP in MV-susceptible transgenic Ifnarko™-CD46Ge mice. The primary objectives were to identify potential toxic side effects and to define the optimal equivalent dosage of MV-sNAP prior to proceeding with a Phase I clinical trial in MBC patients. **Methods:** Ninety-six, 5-to 6-week-old female Ifnarko™CD46Ge mice were stratified into 6 groups with 2 cohorts (48 mice/ cohort). Treatment with MV-sNAP (doses: 10^6 or 10^7 $TCID_{50}$/mL) or vehicle control was started on day 0 using either a single subcutaneous (SC) or intravenous (IV) injection (day 0) or via 3 repeated SC or IV injections on days 0, 14, 28. Body weight and clinical signs of toxicity were monitored daily, and hematology, plasma chemistry, plasma cytokines, gross pathology, and histopathology of major organs were analyzed on days 11, 12, 54 or 56 of the study. The immune response to MV-sNAP was assessed by a NAP-mediated ELISA virus neutralization test. Biodistribution of MV-sNAP was evaluated by qRT-PCR. **Results:** All mice survived to their respective endpoints, with no evidence of

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clinical toxicity. No significant difference between control and treated animals was observed in complete blood counts or plasma chemistry values including liver function at all time points. Low MV genome copy numbers of MV-sNAP were found on Day 11 in the inguinal lymph nodes (ILN) in 2 of 8 animals that received a single SC low dose injection, and in 3 of 8 animals that received a single SC high dose injection. In contrast, a single IV administration of MV-sNAP resulted in significant MV genome copy numbers in most tissues on day 11 in both dose groups. In animals given three SC MV-sNAP injections, the low-dose group exhibited expression only in the spleen and ILN in 1 of 8 animals on day 56. By day 12, a single IV administration of MV-sNAP resulted in a strong early immune response to MV antigens in all mice of both dose groups. High anti-MV titers were maintained by day 54 following three IV injections in all animals of both dose groups. MV-sNAP did not significantly increase circulating levels of pro-inflammatory cytokines. Histopathologic findings following administration of MV-sNAP showed asymptomatic minimal or mild hemorrhage in the lung following SC or IV administration (1-2/8 animals on day 11), and minimal leukocyte infiltration/inflammation at the SC injection site. These modest changes were interpreted to be non-adverse. **Conclusion:** Both SC and IV delivery of MV-sNAP were well tolerated, and no significant toxicity was observed in a relevant (i.e., MV-susceptible) engineered mouse model. This outcome supports the safety of the MV-sNAP platform for oncolytic virotherapy of MBC. Enrollment in a Phase I clinical trial of MV-sNAP in patients with MBC (NCT 04521764) started in November 2020.

9. Validating Secreted IFNβ as an In Vivo Biomarker of Intratumoral Replication of VSV-IFNβ-NIS

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VSV-IFNβ-NIS is an oncoytic vesicular stomatitis virus which is currently being evaluated in multiple clinical trials. Infection of target tissues can be monitored using SPECT or PET imaging to detect expression of the NIS transgene. IFNβ was encoded in the virus to limit its replication in normal tissues and to enhance the inflammatory/ immune response in the infected tumor. However, we demonstrate here in murine models that IFNβ can also serve as a convenient soluble biomarker of VSV-IFNβ-NIS replication in vivo. We first evaluated the baseline level of IFNβ that is produced by innate immune cells upon intravenous administration of VSV. Infusion of 1e8 $TCID_{50}$ VSVhIFNβ-NIS in tumor free C57BL mice induced a transient increase of endogenously secreted mIFNβ, between 32-64 pg/ml at 24h, and is undetectable at 48h. In contrast, the level of hIFNβ produced by VSV-hIFNβ-NIS infected cells in tumor free mice was 500-2000 pg/ ml at 24h but is significantly higher in 5TGM1 tumor bearing animals (1000-65,000 pg/ml). The difference in tumor bearing versus tumor free mice becomes more significant at 48h where the virus continues to replicate and spread in the permissive 5TGM1 tumors. Varying doses of VSV-mIFNβ-NIS was also given intravenously to mice bearing 5TGM1 or MPC11 tumors, and blood levels of IFNβ were measured. Results indicate that the level of virally encoded IFNβ in tumor bearing mice is

dose dependent. There was a corresponding increase in median mIFNβ detected in the plasma of 5TGM1 mice given 1e8, 3e8 and 1e9 $TCID_{50}$ VSV-mIFNβ-NIS. Importantly, the level of IFNβ remained high over days 1-3, and started to decline from day 4, suggesting that secreted IFNβ could be a valuable biomarker to monitor the kinetics of viral infection in cells, followed by subsequent extinction of the infection and death of infected cells. We did not observe a secondary wave of infection in these immunocompetent animals. By day 7, there was no detectable levels of mIFNβ. Corresponding anti-VSV antibody titers are detectable by day 7, and plateaued by day 15. Interestingly, when we compared intratumoral versus intravenous administration of 3e8 $TCID_{50}$ of virus, we found overall higher levels of plasma mIFNβ in the intravenous group versus the intratumoral group, suggesting that systemic delivery of an oncolytic virus might be more beneficial to allow a more uniform seeding of the tumor parenchyma and infectious foci. In the MPC-11 plasmacytoma model in Balb/c mice, we saw a significantly higher level of plasma IFNβ, indicating a much more permissive tumor substrate for viral replication, and/or rapid death and release of cellular contents. In summary, our study confirms that virally encoded IFNβ produced by VSV-IFNβ-NIS infected cells can serve as a convenient and simple biomarker of viral replication in vivo, and that the level and profile (duration) of IFNβ in the plasma could serve as an early indicator of the relative permissiveness of the tumor substrate to the oncolytic virotherapy. These findings in the murine models are recently corroborated by early findings in the intravenous VSV-IFNβ-NIS Phase I trials in cancer patients.

10. Development of Novel Oncolytic Vector Based on Alternative Adenovirus Serotype 6 for Glioblastoma and Breast Cancer Therapy

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The majority of adenovirus oncolytic agents are based on the adenovirus serotype 5 (Ad5), which is the most studied and the most used type of Ad in the field of gene therapy. However, there is a

constant search for alternative Ad types, which are able to overcome the existing obstacles hampering the Ad5 application such as high seroprevalence and rapid clearance of viral particles after i.v. administration. Among many examined alternative Ad serotypes, adenovirus serotype 6 (Ad6) exhibits high oncolytic efficacy against many cancer types. Importantly, Ad6 was shown to have a lower seroprevalence and improved ability to escape from Kupffer cells when compared to Ad5. We recently demonstrated that wild-type Ad6 exhibits a dose-dependent cytotoxicity against glioblastoma cells *in vitro* and a significantly improved therapeutic effect in U87MG xenografts after intratumoral injection, which was comparable of that with Ad5 wild-type control vector. To further enhance the therapeutic efficacy of Ad6, we constructed a tumor-specific recombinant vector Ad6hTERT-GMSCF with the E1 region controlled by the human telomerase reverse transcriptase promoter (hTERT) and the human granulocytemacrophage colony-stimulating factor (GMCSF) transgene expressed from the Ad E3 region. The immunomodulatory activity of secreted GMCSF was evaluated after infection A549 lung adenocarcinoma cells with Ad6-hTERT-GMSCF by assessing the stimulation level of human erythroleukemia cells TF-1 proliferation. The oncolytic efficacy of Ad6-hTERT-GMSCF *in vitro* and *in vivo* was evaluated on both triplenegative breast cancer (MDA-MB-231) and glioblastoma multiforme (U87MG) cell lines. The insertion of hTERT promoter and GMSCF into the Ad6 genome did not significantly affect the oncolytic potential of Ad6 vector as it was demonstrated by the cell viability test. The *in vivo* efficacy of Ad6-hTERT-GMSCF in comparison to wild-type Ad6 and Ad5 vectors was evaluated using subcutaneous MDA-MB-231 or U87MG xenografts in SCID mice after performing injections three times (day 1, 3, and 5). The new recombinant Ad6-hTERT-GMSCF was able to significantly inhibit the tumor growth in both MDA-MB-231 and U87MG models. Importantly, the therapeutic effect of Ad6-hTERTGMSCF was comparable with that of Ad5 and Ad6 wild-type control viruses. We are currently investigating the level of GMCSF in tumor xenografts. The oncolytic potential of a novel Ad6-hTERT-GMCSF vector needs further investigation in immunocompetent models. Further, additional genetic modification (e.g., fiber knob switching) may be beneficial for further enhancement of anticancer efficacy of Ad6-based recombinant vectors.

11. Human Cytomegalovirus Engineered for Glioma Therapy

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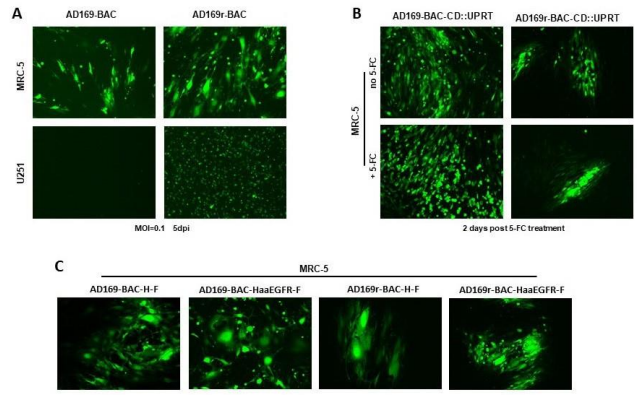
Human cytomegalovirus (HCMV) is a 230nm diameter betaherpesvirus with a ~235kb double-stranded linear DNA genome, an icosahedrally ordered nucleocapsid, a tegument layer and a complex envelope. HCMV naturally hijacks neutrophils and monocyte/ macrophages for local spread, then infects endothelial cells and disseminates systemically in various hematological cell types. In the brain, HCMV infects glial cells and spares neurons. We are therefore interested to develop HCMV as an oncolytic platform for the treatment of glioma since it should infect both the tumor cells and tumor infiltrating macrophages and, if appropriately armed, may efficiently recruit cytotoxic T cells and NK from circulation to target CMV antigen positive cells within the glioma.To develop HCMV as a glioma-targeting vector we worked with the lab adapted strain AD169 and constructed a bacterial artificial chromosome (BAC) clone (AD169-BAC). Genome sequencing confirmed the expected sequence deletions between UL1-UL20 and of the UL/b´ region which in wtCMV encode protein and RNA products that combat T cell and NK cell activation promote virus latency in hematological cells. Vaccines based on lab adapted AD169 and Towne strains have good safety profiles in clinical trials. To enhance glioma cell targeting, we repaired the function of the gH/gL/UL128-131 glycoprotein complex by replacing the UL131 like sequence in the AD169 backbone with the UL131 ORF from Merlin strain (AD169r-BAC). Comparing AD169BAC and AD169r-BAC, both efficiently infected and killed MRC-5 cells, but only AD169r-BAC spread in U251 glioma cell cultures (Fig A). Both viruses showed antitumor activity in U87 and U251 glioma xenograft models, although AD169r-BAC was more potent. We next knocked out the US1-US11 region which encodes proteins and RNAs that inhibit MHC-I and MHC-II pathways and in vivo testing in U87 model suggested superior infectivity of these viruses. We next inserted the suicide gene CD::UPRT (cytosine deaminase fused to uracil phosphoribosyl transferase) into AD169-BAC and AD169rBAC (Fig B), and in parallel inserted untargeted and EGFR-targeted measles F/H glycoprotein complexes into the same backbones (Fig C). We are currently testing the efficacy and mechanisms behind these recombinant AD169 viruses expressing suicide genes or fusogenic F/H complexes in subcutaneous and orthotopic glioma models. Our preliminary data suggest that HCMV could be developed as a unique anti-cancer oncolytic or gene therapy platform, and more therapeutic transgenes will be added.



12. Virulent Velogenic Newcastle Disease Virus Is More Oncolytic Than Attenuated and Lentogenic Newcastle Viruses

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Lentogenic LaSota strain, attenuated AMHA1 strain and AMHA2 virulent Iraqi strain of Newcastle disease virus (NDV) are three

replication-competent, non-recombinant natural oncolytic Newcastle disease viruses evaluated in the current study for their anticancer effects against breast cancer cells. All these strains selectively replicate within tumor cells. AMHA1 is attenuated NDV virus after 7 years of passaging in embryonated chicken eggs, carrying avirulent lentogenic fusion protein motif. Iraqi virulent strain AMHA2 carry virulent F protein cleavage site motif. In vitro cytotoxicity assay, clonogenic assay, replication curve, quantitative real-time PCR assay for NDV mRNA and cytopathic effect studies revealed that virulent AMHA1 strain was more effectively infected, replicated, and killed human and mouse breast cancer cells, followed by attenuated AMHA2 and lastly lentogenic LaSota being less effective. The virulent AMHA2 strains were able to induce more powerful apoptotic response as studied by acridine orange-propidium iodide apoptosis assay in breast cancer cells followed by attenuated AMHA1, in contrast to LaSota NDV strain which produce less apoptotic effect yet still significant. Immunohistochemistry performed on human and mouse breast cancer

cells revealed high NDV HN viral protein expression in cancer cells. To study the apoptosis mechanism induced by each strain, caspase-8 and caspase-9 protein expression were evaluated and found that caspase-9 showing higher expression induced by all three strains with association of caspase-8 which have lower expression. The use of more virulent strains in oncolytic virotherapy can be more efficient strategy for effective tumor treatment.

13. Generation and Characterization of Replication-Competent Oncolytic Foamy Virus Vectors

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Simian Foamy Viruses (SFVs) are ancient, non-pathogenic retroviruses that infect a variety of nonhuman primates with sporadic cases of zoonotic transmission to humans. Similar to Gammaretroviruses replication of SFVs is limited to dividing cells, however, unlike Gammaretroviruses, SFVs have a remarkable ability to persist unintegrated at the centrosome of quiescent cells for weeks, waiting for nuclear membrane disintegration during cell division. This feature enables SFV to efficiently infect cells with long doubling times, such as human tumor cells, which have been reported to have doubling times between 25 to over 200 days. SFV's unique features make it a promising candidate for a useful human cancer therapy. Here, we describe the generation and characterization of oncolytic Simian Foamy Virus (oFV) vectors. oFV was generated by combining genome segments from 2 strains of the chimpanzee SFV - PAN1 and PAN2. oFV was then engineered to carry a reporter gene - GFP in place of the *bel-2* gene (oFV-GFP). Both the parental and the GFP-carrying virus replicated efficiently, albeit slowly, in a panel of human cancer cell lines *in vitro* and exhibited a clear oncolytic activity *in vivo.* A single dose of 10^7 IU of either vector potently controlled growth of orthotopic intraperitoneal ovarian cancer metastases and significantly prolonged survival of the treated mice. Subsequently, we armed oFV with a suicide gene, HSV1 Thymidine Kinase (TK), and tested *in vivo* in subcutaneous xenograft glioblastoma tumors engineered to express firefly luciferase in response to FV infection. Upon infection with oFV-TK and treatment with Ganciclovir, luminescence in the infected indicator tumors decreased, indicating accelerated death of the oFV infected cells. Our data show that oFV is a promising gene delivery platform and candidate for a cancer therapeutic.

14. An Oncolytic Adenoviral Vector Expressing an Anti-PD-L1 scFv Reduces Tumor Growth in a Melanoma Mouse Model

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Oncolytic virotherapy is an emerging therapeutic approach, based on replication-competent viruses, to selectively infect and destroy cancer cells, causing the release of tumor-associated antigens (TAA), therefore stimulating an antitumoral immune response. Indeed, oncolytic adenoviruses (Onc.Ads) can kill cancer cells in different ways, primarily by inducing immunogenic cell death. To increase the anticancer activity of Onc.Ads, it is possible to combine them with a strategy aimed at blocking tumor immune evasion. Programmed death ligand 1 (PD-L1) is mainly expressed on tumor cells surface; binding to its receptor, PD-1, expressed on CD8+ T cell inhibits their proliferation and antitumoral activity. As demonstrated with monoclonal antibodies, a PD-L1 antagonist can prevent the immune escape T cell-mediated of the tumor cells. We developed an Onc.Ad expressing a single-chain variable antibody fragment (scFV) against PD-L1 to combine blockage of PD-1/PD-L1 interaction with the antitumoral activity of Onc.Ads. We confirmed the expression and secretion of scFv anti-PD-L1 in the supernatant of infected cells by western blot analysis. B16-OVA cells (a mouse melanoma cell model) were then infected with Onc.Ad5Δ24scFV-PD-L1 and treated with C57BL/6 splenocytes. We observed that treatment combination was significantly more effective in reducing cell viability. We then evaluated tumor progression *in vivo* in three groups of mice, inoculated with syngeneic B16-OVA melanoma cells, treated with either Onc.Ad5Δ24-scFV-PD-L1, Onc.Ad5Δ24 or mock treatment. We observed that treatment with Onc.Ad5Δ24-scFV-PD-L1was more effective in reducing melanoma growth compared to mice treated with Onc.Ad5Δ24. We will further immunologically characterize immune response induced by the combined treatment; however, these results suggest that Onc.Ad-induced expression of an immune checkpoint inhibitor is an effective and promising strategy.

Delivery Technologies and CRISPR for Therapeutics

15. CRISPR/Cas9-Mediated Targeted Gene Insertion Platform Achieves Durable, Normal Human Alpha-1 Antitrypsin Protein Levels in Non-Human Primates

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Carri Boiselle, Catherine Moroski-Erkul, Kathryn Walsh, Tenzin Yangdon, Elena Kollarova, Vinita Doshi,
Riley Cole, Nikunja Kolluri, Shreelekha Jaligama, Kenneth Manning, Harini Sampath, Dohyun Kim, Palak Sharma, Trisha Das, Samantha Soukamneuth, Sucharitha Parthasarathy, Andrew Whynot, Richard Duncan, Lucinda Shaw, Matthew Roy, Michelle Young,
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CRISPR/Cas9-based genome editing offers the potential to address genetic diseases at their source. Using this technology, we have created a liver-directed, modular genome editing platform to enable rapid therapeutic advancement for genetic diseases with high unmet need, by either reducing a disease-causing protein through knockout editing or enabling production of a functional protein through targeted gene insertion. We have pioneered the use of lipid nanoparticles (LNPs) to enable systemic, transient, and well-tolerated delivery of the CRISPR/ Cas9 genome editing system. Using a hybrid approach combining LNPencapsulated CRISPR/Cas9 components with an adeno-associated virus (AAV) for "promoterless" donor DNA template delivery, we achieve targeted gene insertion resulting in high levels of protein expression *in vivo* in both murine and non-human primate (NHP) models. Here, we illustrate the power of our platform for advancing genome editing to treat alpha-1 antitrypsin deficiency (AATD). In this disease, mutations in the *SERPINA1* gene lead to liver pathology due to aggregation of the alpha-1 antitrypsin (AAT) protein in hepatocytes, and lung pathology due to deficiency of the AAT protein in the lungs. Normal levels of AAT protein in humans are approximately 1,0002,700 ug/mL, a high level that has been challenging to achieve using chronic protein augmentation therapy or traditional gene therapy approaches. By leveraging CRISPR/Cas9 to perform precise gene insertion downstream of the strong endogenous albumin promoter, we now demonstrate the sustained production of normal human levels of AAT protein in NHP, without impacting normal albumin levels. The AAT protein concentration rose quickly after a single administration, reaching the normal range by week 4, and remained stable within the normal range through week 14 in an ongoing study. The physiological levels of human AAT protein produced in this study are expected to be fully therapeutic to restore protease inhibition and protect the lungs in AATD. These results in NHPs build on our previous findings in a humanized mouse model of AATD in which we showed successful consecutive *in vivo* genome editing (*SERPINA1* knockout plus insertion) as an alternative approach to addressing both liver and lung manifestations of the disease. Moreover, they highlight the potential of our modular targeted gene insertion platform to produce durable, high-level expression of therapeutic proteins for diverse genetic diseases, beyond what has been achieved using traditional gene therapy approaches.

16. Direct rAAV-Mediated *In Vivo* Gene Editing of Hematopoietic Stem Cells

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Gene editing of hematopoietic stem and progenitor cells (HSPCs) has progressed to clinical stage and represents a tremendously promising platform for future gene therapy for hemoglobinopathies such as sickle cell disease (Hgb SS disease). However, there are inherent practical limitations to scaling up such approaches to make them accessible to global populations most affected by these disorders. Thus, *in situ* gene modification that enables a direct targeting of the HSPCs *in vivo* would be ideal. Most prior *in vivo* editing techniques involved systemic injection of the editing machinery focused on targeting the liver, thus taking advantage of the efficiency of rAAV-mediated liver gene transfer. In this study, we are employing a targeted delivery strategy of direct injection of rAAV encoding a transgene flanked by homology arms to initiate homology directed repair (HDR) mediated gene editing of HSPCs in the bone marrow. To obtain stable transgene expression without adversely affecting endogenous gene expression, we decided to edit at the genomic safe harbor (GSH) site, AAVS1. First, we optimized the construct *in vitro* to ensure optimal transgene expression in target cells. We compared expression of the reporter GFP expressed by the CMV, EF1a and MND promoters. Human HSCs were isolated from cord blood by negative selection and enrichment of CD34+ cells, followed by electroporation with ribonucleoprotein complex containing AAVS1 guideRNA and Cas9. CD34+s cells were then transduced with rAAV6 expressing GFP from the different promoters flanked by AAVS1 homology arms. The MND promoter reported robust and long-term (up to 9 days) GFP expression in human HSCs *in vitro*. Additionally, the number of GFP expressing cells increased over time in culture suggestive of editing. Based on the *in vitro* results, we shortlisted the MND promoter for subsequent *in vivo* experiments. Since our objective is to target HSCs and cells of the hematopoietic lineage, it is necessary to assess whether the MND promoter is expressed in these cells of interest. To test this *in vivo*, firstly we established conditions for optimum engraftment and differentiation of human CD34+s in the mouse bone marrow. We then engrafted human HSCs, electroporated with CRISPR/Cas editing machinery and transduced with rAAV6 encoding GFP driven by MND promoter, flanked by AAVS1 homology arms into

immunocompromised NBSGW (nonobese diabetic (NOD)-severe combined immunodeficiency (SCID)-gamma) mice. About 40-80% human chimerism (CD45+) and multilineage distribution of HSCs was observed in peripheral blood, bone marrow and spleen of both control and transduced mice. A low frequency of GFP+ cells of the myeloid lineage was obtained in the bone marrow of the mice subjected to editing. Next, to determine HDR-based editing efficiency *in vivo*, we injected rAAV6 encoding AAVS1 homology arms and GFP driven by the MND promoter directly into the bone marrow of engrafted NBSGW mice. Our ddPCR results confirm that a localized intraosseous injection concentrates the vector in the targeted niche, thereby specifically targeting the bone marrow and enhancing transduction of the desired cell types. Future studies using a HDR based editing approach are underway to establish an optimum method for *in vivo* editing of HSPCs.

17. CRISPR-Cas9 Genome Editing of Human CD34+ Cells at Gamma-globin Promoter to Induce Fetal Hemoglobin as Sickle Cell Disease Therapy

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Delivery Technologies and CRISPR for Therapeutics

Remarkably, individuals that co-inherit SCD mutations and genetic variants that cause hereditary persistence of fetal hemoglobin (HPFH) are largely asymptomatic. Thus, a promising genome editing strategy to treat SCD is to induce fetal hemoglobin (HbF, $\alpha 2\gamma 2$) to replace abnormal sickle adult hemoglobin (HbS, $\alpha 2\beta^{s}2$). Recently, disruption of an erythroid-specific enhancer of BCL11A to elevate HbF ($\alpha 2\gamma 2$) was reported to be promising in an early clinical trial (Frangoul et al.). Another promising approach is to disrupt repressor-binding motifs for BCL11A or ZBTB7A proteins in the γ-globin gene promoters in HSCs.To compare the efficiency of editing BCL11A and ZBTB7A binding sites in the γ-globin gene (*HBG1* and *HBG2*) promoters and associated levels of HbF ($\alpha 2\gamma 2$) induction, we electroporated human primary $CD34^+$ hematopoietic stem and progenitor cells (HSPCs) with Cas9-3xNLS ribonucleoproteins (RNPs). We observed high editing efficiencies (83.8%-97.9% indels) and transplanted edited HSPCs into immunodeficient NBSGW mice. 17 weeks post-transplantation, all hematopoietic lineages derived from RNP-treated donor HSPCs exhibited 63.5 to 92.7% indel mutations at the γ-globin promoter ZBTZ7A or BCL11A binding sites, indicating consistent, high-level editing of repopulating hematopoietic stem cells (HSCs). Editing of the BCL11A binding site resulted in HbF induction up to 31.8% in erythroid progeny, compared to <2% in erythroid progeny from unedited HSCs. Disruption of the ZBTB7A binding site at similar frequencies also resulted in erythroid HbF induction, although to a lesser extent (13-18%).To assess our approach in SCD patient cells, we edited plerixafor-mobilized $CD34^+$ HSPCs from one healthy donor and three adult individuals with SCD using Cas9-3xNLS RNPs targeting the BCL11A binding site in the γ-globin promoter. We attained consistently high indel rates ranging from 80.6% to 94.5% in both $CD34^+/CD90^-$ progenitor and $CD34^+/CD90^+$ HSC-enriched populations. 17 weeks following xenotransplantation of edited cells, we observed persistent high-level editing (49.3%-91.5%) of all HSC-derived lineages with HbF levels of 18.3% to 34.3% in human erythroid progeny compared to <5% in unedited controls. Single cell western blot revealed broad HbF induction, where 49-58% of edited erythroblasts showed γ-globin expression compared to controls (<6%). One concern is that Cas9directed double strand breaks in the γ-globin promoters could result in the deletion of the intervening 4.9-kb region. By digital droplet PCR, we observed 20-25% frequencies of this 4.9 kb deletion in HSPCs prior to xenotransplantation and at 17 weeks post-engraftment. We are currently validating unintended genome wide activities identified by CHANGE-seq and *in silico* methods. In conclusion, our preclinical data suggests that *ex vivo* modification of autologous HSPCs via CRISPRCas mediated disruption of the BCL11A repressor binding site in the gamma-globin promoter genes can induce HbF to therapeutically relevant levels, and therefore, represents a promising genome editing cell therapy for SCD.

Delivery Technologies and CRISPR for Therapeutics

18. In Utero Lipid Nanoparticle Delivery of CRISPR Technology to Correct Hereditary Tyrosinemia Type 1

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According to the WHO, ~295,000 newborns die within 28 days of birth every year from congenital diseases many of which have a genetic origin. Many of these diseases can be diagnosed prenatally

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Sickle cell disease (SCD) affects nearly 100,000 Americans and millions of individuals worldwide. Patients with SCD are affected by pain crises, chronic anemia, multi-organ dysfunction, and early mortality.